Int J Pharm Bio Sci 2017 Apr ; 8(2): (B) 553-561

Original Research Article Molecular Biology

International Journal of Pharma and Bio Sciences

ISSN 0975-6299

ROLE OF NF-κB TRANSCRIPTION FACTORS, CELL-CYCLE REGULATORS AND APOPTOTIC GENES IN PROLIFERATION OF MCF-7 CELLS

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ABSTRACT

The cross-talk between steroid hormones and their classical nuclear receptors (NRs) play a major role on the development and progression of breast cancer. In a non-classical genomic pathway, hormone-receptor complex tether to transcription factors (TFs) such as activator protein-1 (AP-1) or nuclear factor-kappa B (NF-κB) and mediate transcription of target genes involved in breast cancer progression. MCF-7 cells treated with steroid hormones alone/in combination and steroid hormones in combination with their antagonists were used as *in vitro* model system in the present study. Direct cell counting assay showed that E2 increased cell proliferation with time. The *semi q*RT-PCR showed that steroid hormones alone increased the mRNA levels of NF-κB TFs, Cyclin D1, Cyclin E1, E2F1 and Bcl2 by more than 1-fold and decreased p53, p21 and Rb mRNA levels by more than 20%. The steroid hormone antagonists ICI 182,780 and RU486 decreased the mRNA levels of NF-κB TFs, Cyclin D1, Cyclin E1, E2F1 and Bcl2 by more than 35% and increased the levels of mRNAs of p53 and Rb by 1-fold and Bax by more than 26%. Results of the present study suggested that steroid hormones probably tether to NF-κB TFs and upregulate cyclins and E2F1 genes which culminate in the increased proliferation. Thus ERα/NF-κB/E2F1 pathway serves as an important target in the therapeutic modality for treating triple positive breast cancer cells.

KEYWORDS: Steroid hormones, nuclear receptors, *semi q*RT-PCR, NF-κB TFs, cell cycle regulators, apoptotic genes

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Received on: 13.2.2017
Revised and Accepted on: 24-03-2017
DOI: http://dx.doi.org/10.22376/ijpbs.2017.8.2.b553-561

INTRODUCTION

Steroid hormones play a major role in the growth and development of breast epithelial tissues through the interaction with their specific nuclear receptors. Breast cancer is a type of adenocarcinoma caused due to the over-expression of nuclear receptors mediated by genomic and non-genomic signal transduction pathways. In a non-genomic pathway, steroid hormone-nuclear receptor complex tether to transcription factors such as AP-1 and NF-κB to regulate various cellular processes such as cell proliferation, inflammation, cell cycle progression and apoptosis. NF-κB TFs containing conserved stretch of 300 amino acids, designated as the Rel homology domain (RHD) are expressed ubiquitously in the cytoplasm and control DNA binding dimerization, interaction with inhibitory factors (I-κB , Inhibitors of kappa B proteins) and different Rel family members.[1, 2] The major types of NF-κB TFs present in mammalian cells include p50/p105 (NF-κB1), p52/p100 (NF-κB2), c-Rel, Rel-B, and p65 (Rel-A). NF-κB TFs have a complex role in cancer and shown to be involved in the control of programmed cell death (PCD), cell-cycle progression, cell differentiation, angiogenesis and cell migration.[3] The levels of NF-κB TFs were found to be increased in various types of cancers including breast cancer. The increased NF-κB activity in breast cancer was found to be associated with resistance to apoptosis, and with this, the cells acquire the ability to grow in the absence of estrogen.[4] Thus, NF-κB signaling plays a major role in the regulation of breast cancer cell growth and the inhibition of NF-κB expression may induce sensitivity of cancer cells to anti-steroidal drugs. Cell cycle plays an important role in the growth and development of breast cancer. Cyclins and cyclin dependent kinases (CDKs) are the important factors that regulate cell cycle progression or arrest. Breast epithelial cells receive signals from various factors such as growth factors (TGF, EGF, and VEGF etc), steroid hormones, cytokines and cell matrix which converge on pRb inactivation depending on the levels of cyclins and CDK inhibitors and thus regulate G1/S transition. Cyclin D and cyclin E families of cyclins are mainly regulated by mitogens such as steroid hormones, phorbol ester etc. Earlier studies showed that cyclin D1 is a critical modulator of G1/S transition through Rb phosphorylation and p21/p27 titration and activation of cyclin D1–CDK4/6 complex involves CAK-mediated phosphorylation. Foster et al., (2001) and Cicatiello et al., (2004)[5-7] have shown that cyclin D1 is activated by estrogen via AP-1 or Sp1 since cyclin D1 does not contain estrogen response element (ERE) site in its promoter region. The retinoblastoma (Rb) gene is a tumor suppressor gene generally present as a complex with E2F1, an oncogenic transcription factor, and serves as an important substrate for CDK4/CDK6 binding which when hypo-phosphorylated, separates from E2F1 and activation of E2F1 which acts as an important target for cyclin E, drives the expression of cyclin E to reach maximum level. The activated cyclin E-CDK2 complex phosphorylates pRb, and provides a positive feedback loop mechanism for the transcription of cyclin E. The inhibition of cyclin E-CDK2 complex by p21 and p27 depends on their sequestration by cyclin D1-CDK4/6 and by cytoplasmic relocalization.[9,10] Anti-steroidal drugs such as fulvestrant (ICI 182,780 an anti-estrogen) and Mifepristone (RU486, an anti-progesterone and anti-glucocorticoid) are used in the treatment of breast cancer. ICI 182,780 acts as ER-analogue and competes with estrogen to bind to classical ER-α and down-regulates ER-α mediated transcription through competitive inhibition. Studies showed that RU486 binds to PR and GR and brings about downregulation of PR and GR mediated transcription of genes and also could function independent of PR or GR. These drugs have cytostatic effect and bring about changes at cellular and molecular levels by inhibiting NF-κB activity, inducing G0-G1 arrest and apoptosis in breast cancer cells.[11, 12] Previously we have shown that classical nuclear receptors (ER, PR and GR) are involved in the regulation of AP-1 transcription factors and thereby influence the growth progression of cancer cells.[13] In spite of several reports on the involvement of transcription factors, cell cycle regulators and apoptotic genes modulating breast cancer, the mechanism of cross-talk of steroid hormones and their classical receptors in the regulation of NF-κB transcription factors, cell cycle regulators and apoptotic genes remains unclear. In the present study, an attempt has been made to treat MCF-7 cells with steroid hormones (estrogen, progesterone and glucocorticoid) and their specific antagonists (ICI 182,780 and RU486) to study the expression pattern of mRNAs of NF-κB TFs, cell cycle regulators and apoptotic genes.

MATERIALS AND METHODS

Materials

Human breast cancer cells (MCF-7) were purchased from NCCS (Pune, India); Estradiol-17β (E2), Progesterone (P4), Dexamethasone (Dex), Fulvestrant (ICI 182,780), Mifepristone (RU486), forward and reverse primers for NF-κB transcription factors, different cell cycle regulators, and apoptotic genes were purchased from Sigma-Aldrich (St Louis, USA). Superscript reverse transcriptase for RT-PCR was purchased from Invitrogen (CA, USA). RPMI 1640 media, fetal bovine serum (FBS), penicillin, streptomycin, glutamine, dimethyl sulfoxide (DMSO), Sodium Pyruvate, and Trypan blue dye were purchased from Himedia (Mumbai, India). Taq DNA polymerase (1 U/μl) was procured from Merck-Millipore (Mumbai, India).

Cell culture and treatment regimen

MCF-7 cells were grown and sub-cultured as described earlier. [13] Briefly, MCF-7 cells were grown in 75 cm^2 culture flask in phenol red free RPMI 1640 media supplemented with 10 % FBS, antibacterial and antimycotic agents, 2 mM L-glutamine, and 1 mM sodium pyruvate. Cells were cultured in a humidified atmosphere at 37 °C by passing 5 % CO_2 in an incubator. On arriving at confluence, the cells were sub-cultured in 6-well plate (5×10^5 cells/well) for the treatment with steroid hormones alone and in combination or steroid hormones in combination with their specific antagonists.

Cell count by trypan blue exclusion method

MCF-7 cells (3×10^5 cells/well) were seeded in different cell culture dishes and after the attainment of 75% confluence, the cells in each dish were treated with E2 (100 nM) and further incubated for 0-96 h. The cells in each culture dish were washed with PBS, trypsinized and mixed with trypan blue dye and used for counting using Neubauer counting chamber. The cells counted at 0 h were considered as control.

RNA isolation and semi-quantitative reverse transcription PCR (semi qRT-PCR)

MCF-7 cells (3×10^5 cells/well) seeded in a 6-well plate and cultured overnight were treated with E2 (100 nM), P4 (150 nM), Dex (100 nM), E2+P4 (100:150 nM), E2+Dex (100:100 nM), P4+Dex (150: 100 nM), E2+P4+Dex (100:150:100 nM), E2+ICI 182,780 (100:10 nM), P4+RU486 (100:10 nM), Dex+RU486 (100:10 nM) for 48 h. Total RNA was isolated from each of the samples using ''Trizol reagent'' following manufacturer's instructions. Total RNA (2 μg) was reverse transcribed using Oligo (dT) primers and superscript reverse transcriptase as described by Sharma et al (1999).[14] The cDNA was subjected to 30 cycles of PCR using different forward and reverse primers of NF-κB transcription factors, different cell cycle regulators [Table 1-2], apoptotic genes and β-actin[15] using appropriate annealing temperatures in a gradient thermocycler (Eppendorf, USA). Amplicons were analyzed on 1 % agarose gel using 1X TAE buffer. Relative mRNA levels were quantified using image analysis software (ImageJ). The expression of β-actin mRNA was used as a positive control and for normalization.

Table 1
Sequence of primers used for the amplification of NF-κB transcription factors

Gene	Primer Sequence (5′→ 3′)	Annealing Temp. (°C)	Product size (bp)
NF-κB TFs			
NF-κB1	F: CTGTCAGTGGGTGCCAGTAG R: ACCGCATGACTCTATCAGCG	60	280
NF-κB2	F: ACTAAGGTCGGCCATCATTCC R: TATCCCTGAGAATGGGGCCC	62	282
Rel-A	F: AGGCTATCAGTCAGCGCATC R: TGTATTTCACGGGACCAGGC	58	290

Columns 3 and 4 show annealing temperatures and the size of the amplified products. F: forward, R: reverse

Table 2
Sequence of primers used for the amplification of cell cycle regulators

	Primer Sequence (5′→ 3′)	Annealing Temp.(°C)	Product size (bp)
Cell Cycle Regulators			
CDK4	F: AGTGGCGGATCCATGGCTACCTCTCGATAT R: TCTCGGAAGCTTTCACTCCGGATTACCTTCA	60	912
Cyclin D1	F: AGACCTGCGCGCCCTCGGTG R: GTAGTAGGACAGGAAGTTGTTG	58	574
Cyclin E1	F: GTCCTGGCTGAATGTATACATGC R: CCCTATTTTGTTCAGACAACAT	60	415
p53	F: GAGCCCCCTCTGAGTCAG R: GCAAAAACATCTTGTTGAG	56	375
p21	F: GATCACAAGCAGTGGGGTGA R: CTGAGTGACTGCACGACCTT	58	160
Rb	F: AAGTACCCATCTAGTACT R: AAGTTACAGCATCTCTAAA	52	547
E2F1	F: TGCAGAGCAGATGGTTATGG R: ATCTGTGGTGAGGGATGAGG	62	265

Columns 3 and 4 show annealing temperatures and the size of the amplified products. F: forward, R: reverse

RESULTS

Effect of estradiol-17β on the cell proliferation

MCF-7 cells treated with E2 (100 nM) were incubated for different periods (0 - 96 h) and the cells without E2 treatment were considered as control. The total number of cells was calculated for 24, 48, 72, and 96 h. The results presented in Fig. 1 shows that E2 stimulated proliferation up-to 96 h which increased significantly by 3- and 3.2-fold at 48 and 72 h respectively when compared to control.

Effect of steroid hormones and their antagonists on the expression of NF-κB transcription factors

MCF-7 cells were treated with steroid hormones alone and in combination with their specific antagonists (E2 / E2+ICI 182,780 / P4 / P4+RU486 / Dex / Dex+RU486) for 48 h. The cells without any treatment were considered as control. The results show the expression pattern of mRNAs of NF-κB TFs. The cells treated with E2 alone showed a significant increase in the levels of mRNAs of Rel-A and NF-κB2 by 1.7- and 2-fold respectively and decrease the mRNA level of NF-κB1 by more than 60% when compared to control. The cells treated with P4 alone showed a significant increase in the level of mRNAs of Rel-A by 2-fold, NF-κB1 by 10% and NF-κB2 by 2.7-fold when compared to control. The cells treated with Dex alone increased the mRNA levels of Rel-A and NF-κB2 by 2.3-, and 3- fold respectively when compared to control. The cells when treated with steroid hormones in combination with their antagonists decreased the levels of mRNAs of all the three NF-κB

TFs. MCF-7 cells treated with E2+ICI 182,780 decreased the levels of mRNAs of Rel-A, NF-κB1 and NF-κB2 significantly by 90%, 10% and 40% respectively compared to the cells treated with E2 alone. The cells treated with P4+RU486 decreased the levels of mRNAs of Rel-A, NF-κB1 and NF-κB2 by 41%, 48% and 50% respectively compared to the cells treated with P4 alone. MCF-7 cells when treated with Dex+RU486 showed that the mRNAs levels of Rel-A, NF-κB1 and NF-κB2 were decreased significantly by 80%, 30% and 55% when compared to the cells treated with Dex alone. The above results suggest that RU486 may act potentially with Dex than P4 in decreasing the mRNA levels of the NF-κB transcription factors (Fig. 2).

Dex antagonizes the actions of E2 and P4 in the regulation of cell cycle regulators

MCF-7 cells were treated with steroid hormones alone and in combination (E2 / P4 / Dex / E2+P4 / E2+Dex / P4+Dex / E2+P4+Dex) or steroid hormones in combination with their specific antagonists (E2 / E2+ICI 182,780 / P4 / P4+RU486 / Dex / Dex+RU486) for 48 h, and the differential expression pattern of mRNAs of cell cycle regulators were measured by semi *q*-RT-PCR and the results were analyzed. The cells without any treatment were considered as control. Results on the synergistic effects of steroid hormones showed that the cells treated with E2 alone and Dex alone decreased the expression of CDK4 mRNA by 50% and 12% respectively, while P4 alone showed 10% increase in CDK4 mRNA levels compared to control. The cells when treated with E2+P4 show 10% increase in the CDK4 mRNA level compared to control. The cells when treated with E2+Dex and P4+Dex decreased CDK4 mRNA levels by 10% and 61% respectively when compared to the cells treated with E2 alone and P4 alone, while, the cells treated with E2+P4+Dex showed CDK4 mRNA levels similar to that of E2+P4 treated cells. The cells treated with E2, P4 and Dex alone showed a significant increase in the levels of mRNAs of cyclin D1 by 1.4-, 1.3 and 1.3-fold and cyclin E1 by 1.9-, 5- and 5-fold respectively when compared to control. The cells treated with E2+P4 showed a decrease in cyclin D1 mRNA level by 20% and significant increase in cyclin E1 mRNA level by 5-fold when compared to control. The cells treated with E2+Dex showed a significant decrease in cyclin D1 mRNA level by 70% and showed less or no change in the expression of cyclin E1 mRNA level when compared to the cells treated with E2 alone. The cells treated with P4+Dex showed a marginal decrease in cyclin D1 mRNA level and more than 50% decrease in cyclin E1 mRNA level when compared to the cells treated with P4 alone. The cells treated with E2+P4+Dex showed an increase in cyclin D1 expression similar to that as the cells treated with E2 alone and showed 18% decrease in the cyclin E1 mRNA level when compared to the cells treated with E2 alone (Fig. 3). Figure 4 shows the variable effects of steroid hormone antagonists on the mRNA levels of cyclins and CDK4 in MCF-7 cells. The cells treated with E2+ICI 182,780 increased the mRNA level of CDK4 by 20% and decreased the levels of mRNAs of cyclin D1and cyclin E1 by 27% and 50% respectively when compared to the cells treated with E2 alone. The cells treated with P4+RU486 decreased the levels of mRNAs of CDK4, cyclin D1 and cyclin E1 by 30%, 10% and 70% respectively when compared to the cells treated with P4 alone. The cells treated with Dex+RU486 showed an increase in the level of CDK4 mRNA by 1.0-fold and decrease in the levels of mRNAs of cyclin D1 by 10% and cyclin E1 by 56% when compared to the cells treated with Dex alone. The levels of mRNAs of CDK4, Cyclin D1 and Cyclin E1 treated with E2, P4, and Dex alone were found to be as that in Fig. 3. The above results suggested that the activation of Cyclin D1 by E2, P4 and Dex is independent CDK4 binding and may involve binding with CDK6 and increased the cell proliferation. Results in the Fig. 5 depict the variable effects of cross-talk of steroid hormones on the mRNA levels of cell-cycle regulators in MCF-7 cells. The cells treated with E2 alone showed a significant decrease in the mRNA levels of p53 by 42%, p21 by 20% and Rb by 49% and increase in the level of E2F1 mRNA by 2-fold when compared to control. The cells treated with P4 alone increased the level of mRNAs of p53 by 20%, p21 by 1-fold and decreased the levels of mRNAs of Rb and E2F1 by 31% and 9% respectively when compared to control. The cells treated with Dex alone showed the decreased levels of mRNAs of p53 by 10%, Rb around 30% and E2F1 by more than 50% and no change in the expression of p21 mRNA level when compared to control. The cells when treated with E2+P4 was found to decrease the mRNA levels of p53, Rb and E2F1 by 35%, 38% and 44% respectively, and increase the level of p21 mRNA by 1.3-fold when compared to control. When the cells treated with E2+Dex, p53 mRNA level was found to be increased by 20% and E2F1 mRNA level was decreased by 50% significantly with less or no change in the Rb mRNA level when compared to the cells treated with E2 alone. Similarly, the cells treated with P4+Dex showed an increase in the level of the mRNAs of p53 by 30%, Rb by 20% and E2F1 by 25% when compared to the cells treated with P4 alone. However, p21 mRNA level was considerably decreased in the cells treated with E2+Dex and P4+Dex when compared to the cells treated with E2 alone and P4 alone. When the cells treated with E2+P4+Dex, it was found that the mRNA levels of p53 and E2F1 were decreased by nearly 40% and p21 and Rb were found to be increased by more than 1-fold when compared to E2+P4 treated cells. Results in the Fig. 6 show the varied effects exhibited by the steroid hormone antagonists on the mRNA levels of cell cycle regulators in MCF-7 cells. The cells treated with E2+ICI 182,780 significantly increased the mRNA levels of p53 and Rb by 1.0-fold and decreased the levels of mRNAs of p21 and E2F1 by nearly 50% when compared to the cells treated with E2 alone. The cells treated with P4+RU486 showed an increase in the levels of mRNAs of p53 by 1.0-fold and Rb by 14%, and decreased the level of mRNAs of p21 by 70%, and E2F1 by 5% when compared to the cells treated with P4 alone. Similarly, when the cells treated with Dex+RU486, it was found that p53 mRNA level was increased by 2-fold and E2F1 mRNA level by 1.8-fold and decreased the levels of mRNAs of p21 by 17% and Rb by 40% when compared to the cells treated with Dex alone (**Fig. 6**). The levels of mRNAs of p53, p21, Rb and E2F1 were found to be similar to that in **Fig. 5** when the cells treated with steroid hormones alone. The results implicate that E2 and P4 exert similar effects while Dex antagonizes the

effects of E2 or P4 in the regulation of cell cycle regulators. Similarly, ICI 182,780 was found to be a potent antagonist of E2 and RU486 was found to antagonize Dex effects more than that of P4.

Differential effects of Dex on E2- and P4- regulated apoptotic mRNAs

To study the combinatorial effect of steroid hormones, MCF-7 cells were treated with steroid hormones alone and in combination (E2 / Dex / P4 / E2+Dex / P4+Dex) / steroid hormones alone and in combination with their specific antagonists (E2 / E2+ICI 182,780 / P4 / P4+RU486 / Dex / Dex+RU486) for 48 h, and the differential expression pattern of mRNAs of anti- and pro-apoptotic genes were studied by semi *q*- RT-PCR, and relative mRNA levels were analyzed. The cells without any hormone treatment were considered as control. Results presented in **Fig. 7** showed that mRNA level of Bcl-2 was found to be increased by more than 1.37- and 1.33-fold in the cells treated with E2 alone and P4 alone respectively when compared to control. The Bcl-2 mRNA level was significantly decreased by 26% when the cells treated with E2+Dex compared to the cells treated with E2 alone. The Bax mRNA levels were not influenced by any of the steroid hormones used in the study. Results depicted in the Fig. 8 show that mRNA levels of Bcl2 and Bax were found to be modulated when treated with steroid hormones alone and steroid hormones in combination with their antagonists. The mRNA level of Bcl2 was found to be increased significantly as in Fig. 7 in the cells treated with E2 alone and P4 alone when compared to control and decreased by 35% and 25% in the cells treated E2+ICI 180,782 and P4+RU486 respectively when compared to the cells treated with E2 alone and P4 alone. Though the mRNA level of Bcl2 was found to be increased in Dex alone treated cells by 10% compared to control, it was found to be increased up to 1-fold in the cells treated with Dex+RU486 when compared to the cells treated with Dex alone. The levels of Bax mRNA were increased by 26% in the cells treated with E2+ICI 182,780 and 16% in the cells treated with P4+RU486 respectively when compared to the cells treated with E2 alone and P4 alone. Further, the level of Bax mRNA was significantly decreased by more than 75% in the cells treated with Dex+RU486 when compared to the cells treated with Dex alone. The results obtained in the study suggest that E2 and P4 were found to exert synergistic effects while Dex antagonizes the effects of E2 and P4 in the regulation of apoptotic genes.

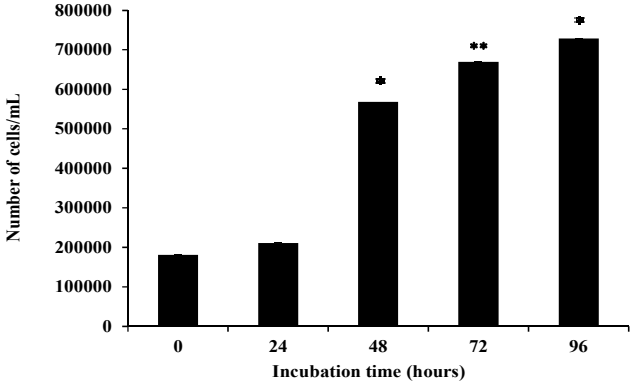

Figure 1

Effect of E2 (100 nM) on the cell proliferation with time

Data were presented as mean ± SD, n = 6. Values were significantly different in figure from the control if *P< 0.05 and **P< 0.005, using student's t-test.

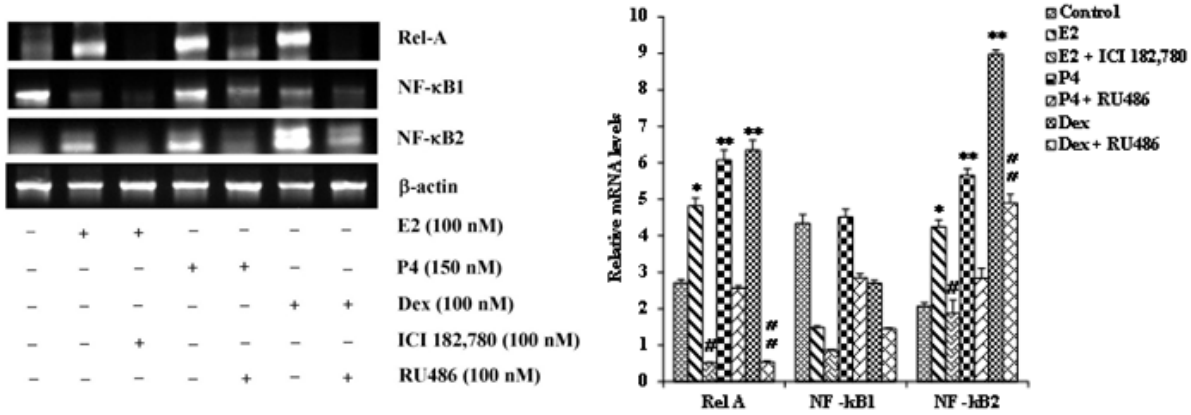

Figure 2

Effect of steroid hormones alone and in combination with their antagonists on the expression of mRNAs of NF-κB transcription factors in MCF-7 cells.

Data presented as mean ± SD, n = 3. Values were significantly different in figure from the control if *P< 0.05 and **P< 0.005, from E2 alone if #P< 0.005, from Dex alone if ##P< 0.005 using one-way ANOVA followed by post hoc Tukey test. The results were shown as representative of three independent experiments

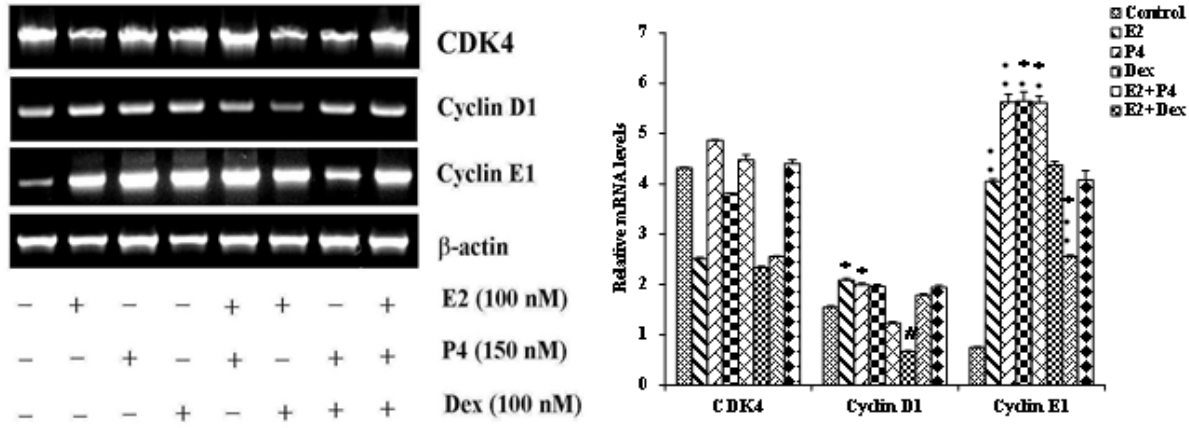

Figure 3
Effect of steroid hormones alone and in combination (E2 / P4 / Dex / E2+ P4 / E2+Dex / P4+Dex / E2+P4+Dex) on the levels of mRNAs of cell cycle regulators in MCF-7 cells.

Data presented as mean ± SD, n = 3. Values were significantly different in figure from the control if $^{*}P<0.05$ and $^{**}P<0.005$, from E2 alone if $^{\#}P<0.005$, from P4 alone if $^{***}P<0.005$ using one-way ANOVA followed by post hoc Tukey test. The results were shown as representative of three independent experiments

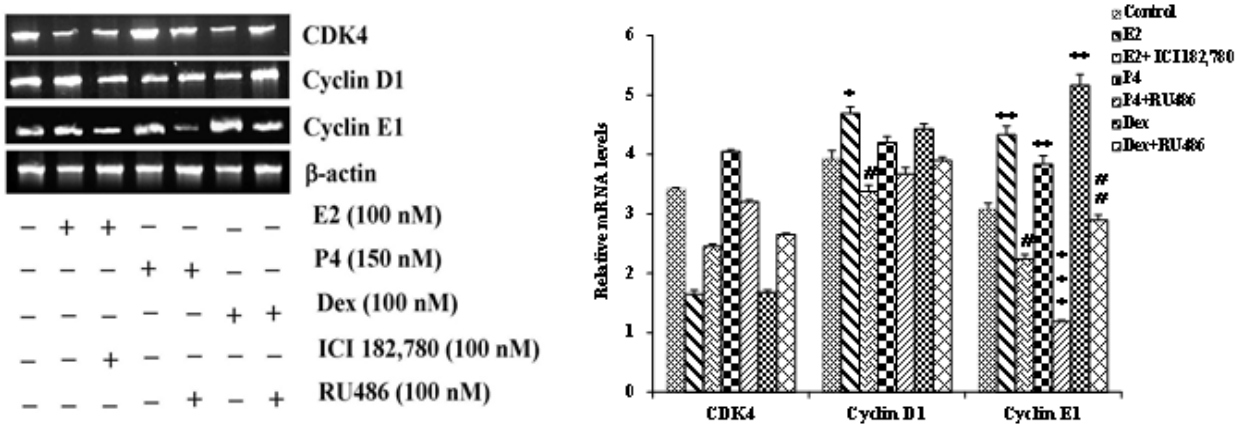

Figure 4
Effect of Steroid hormones alone and in combination with their antagonists (E2 / E2+ICI 182,780 / P4 / P4+RU486 / Dex / Dex+RU486) on the levels of mRNAs of cell cycle regulators in MCF-7 cells

Data presented as mean ± SD, n = 3. Values were significantly different in figure from the control if $^{**}P<0.005$, from E2 alone if $^{\#}P<0.005$, from P4 alone if $^{***}P<0.005$ and from Dex alone if $^{\#\#}P<0.005$ using one-way ANOVA followed by post hoc Tukey test. The results were shown as representative of three independent experiments

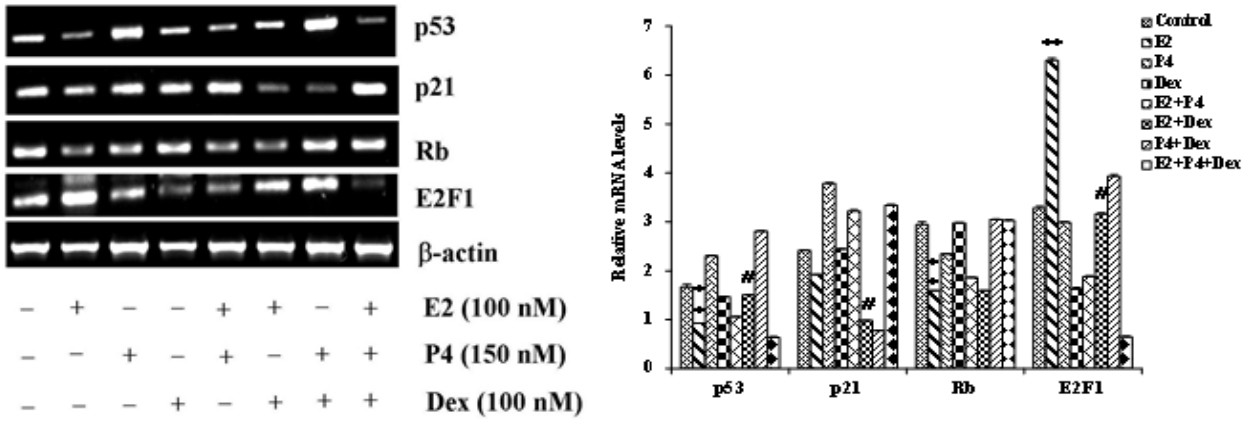

Figure 5
Effect of steroid hormones alone and in combination (E2 / P4 / Dex / E2+ P4 / E2+Dex / P4+Dex / E2+P4+Dex) on the levels of mRNAs of cell cycle regulators in MCF-7 cells

Data presented as mean ± SD, n = 3. Values were significantly different in figure from the control if $^{**}P< 0.005$, from E2 alone if $^{\#}P<0.005$, from using one-way ANOVA followed by post hoc Tukey test. The results were shown as representative of three independent experiments.

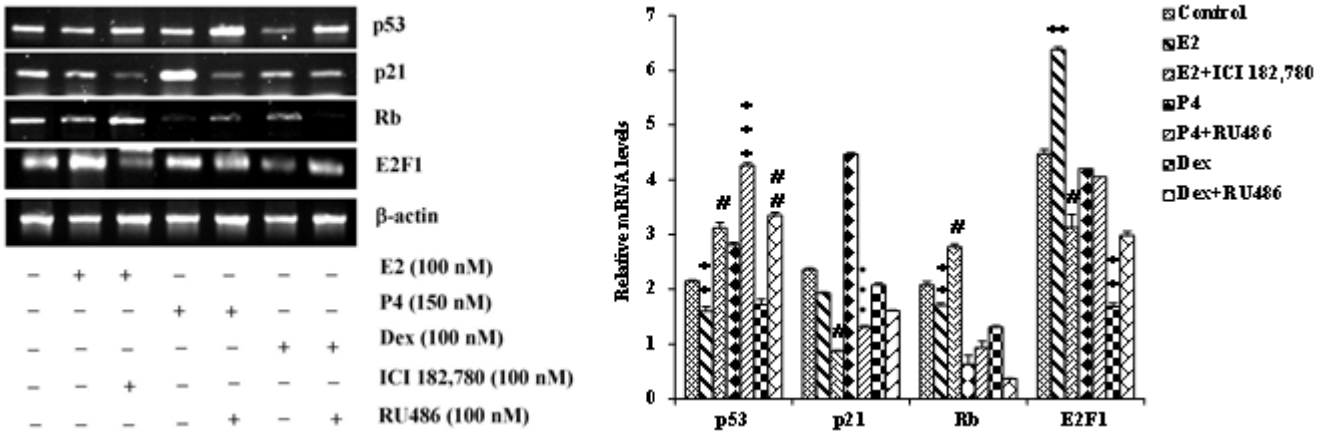

Figure 6

Effect of Steroid hormones alone and in combination with their antagonists (E2 / E2+ICI 182,780 / P4 / P4+RU486 / Dex / Dex+RU486) on the levels of mRNAs of cell cycle regulators in MCF-7 cells

Data presented as mean ± SD, n = 3. Values were significantly different in figure from the control if $^{**}P<0.005$, from E2 alone if $^{\#}P<0.005$, from P4 alone if $^{***}P<0.005$ and from Dex alone if $^{\#\#}P<0.005$ using one-way ANOVA followed by post hoc Tukey test. The results were shown as representative of three independent experiments.

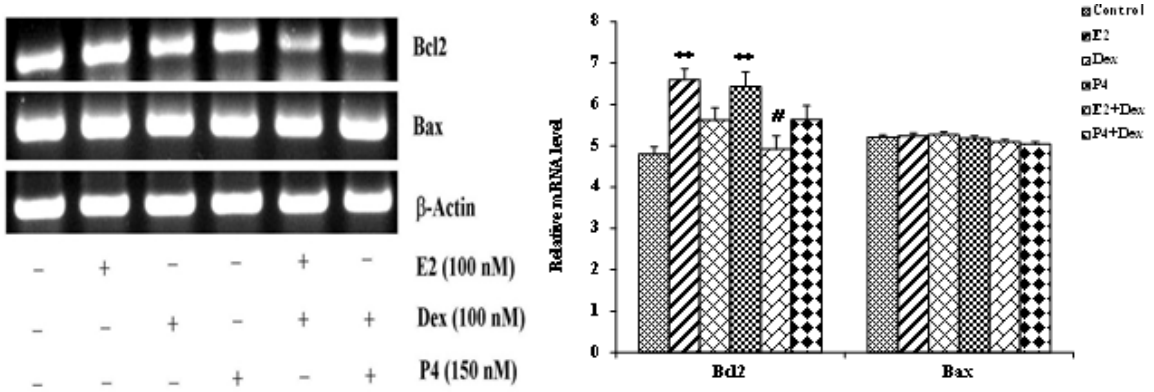

Figure 7

Effect of steroid hormones alone and in combination (E2 / Dex / P4 / E2+Dex / P4+Dex) on the levels of apoptotic mRNAs.

Data presented as mean ± SD, n = 3. Values were significantly different in figure from the control if $^{**}P<0.005$ and from E2 alone if $^{\#}P<0.005$, using one-way ANOVA followed by post hoc Tukey test. The results were shown as representative of three independent experiments.

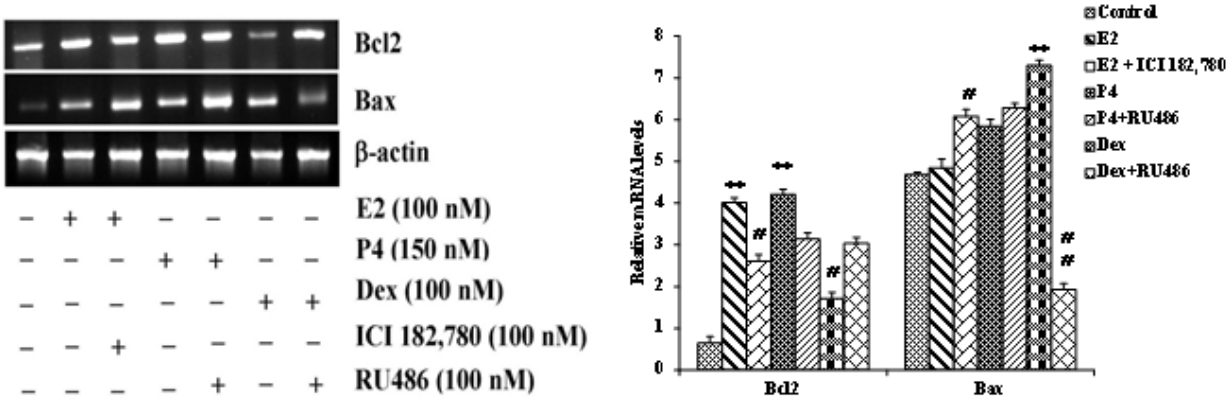

Figure 8

Effect of steroid hormones alone and in combination with their antagonists (E2 / E2+ICI / P4 / P4+RU486 / Dex / Dex+RU486) on the levels of apoptotic mRNAs in MCF-7 cells.

Data presented as mean ± SD, n = 3. Values were significantly different in figure from the control if $^{**}P<0.005$ and from E2 alone if $^{\#}P<0.005$, and from Dex alone if $^{\#\#}P<0.005$ using one-way ANOVA followed by post hoc Tukey test. The results were shown as representative of three independent experiments.

DISCUSSION

Sex steroids, glucocorticoid and their receptors interplay with each other to regulate transcription of many genes involved in breast cancer progression. MCF-7 cells are triple positive breast adenocarcinoma cells which express all the three hormonal nuclear receptors (ER, PR and GR) and their proliferation increases with estrogen or progesterone treatment. [13] Though, several reports describe the role of estrogen, progesterone and glucocorticoid in regulating the breast cancer, the mechanism of their cross-talk in the regulation of cell cycle and apoptosis have not been clearly elucidated. Hence the present work was undertaken to study the role of cross-talk of steroid hormones and their receptors in the expression of NF-κB transcription factors, cell cycle and apoptotic genes in MCF-7 cells. NF-κB is one of the major families of transcription factors involved in breast cancer progression which generally present as a complex with I-κB factors. Various stimuli such as steroid hormones, inflammatory modulators degrade I-κB and activate NF-κB which then translocates into the nucleus and transactivate the target genes involved in proliferation. Increase in the level of transcription factors such as NF-κB or AP-1 by steroid hormones increases the risk of hormone-dependent breast cancer and blocking of TFs by steroid hormone antagonists decrease the cancer progression. In the present study, MCF-7 cells treated with steroid hormones such as E2, P4 and Dex showed increased levels of mRNAs of NF-κB1 and Rel-A TFs and their antagonists such as ICI 182,780 and RU486 subsequently decreased the levels of NF-κB mRNAs. This supports the studies of Karin et al., (2002) [16] who showed that NF-κB-inhibitors-induced-apoptosis was by blocking NF-κB with the increase in p53 mRNA levels and activating the autophagic mechanism in breast cancer cells. The progression of cell cycle through G1 phase is mediated by cyclin D1-CDK4/6 complex and their association is prevented by CDK-inhibitors such as p21 and p27. [17] The present study showed that steroid hormones responsive MCF-7 cells treated with E2, P4 and Dex showed an increase in the levels of cyclin D1, cyclin E1 and decrease in the level of CDK4 mRNAs suggesting that cyclin D1 in this case is probably combining with CDK6 and increased proliferation. Further the cross-talk effects of estrogen and progesterone were studied by treating MCF-7 cells with E2+P4 which showed that E2 and P4 together increased cyclin D1 and cyclin E1 mRNAs with decrease in the levels of p21 mRNAs. These findings are in accordance with the earlier reports. [5, 18, 19] Functional p53 modulates CDK-inhibitor p21, tumor suppressor Rb and oncogenic E2F1 mRNAs which are involved in the regulation of cell cycle arrest. Results obtained in the present study showed the variable expression pattern of p21 at physiological concentrations of steroid hormones. MCF-7 cells treated with E2 alone and Dex alone decreased the levels of p21 mRNA, but the cells treated with P4 alone or E2+P4, showed increased levels of p21 mRNA suggesting that p21 binds and stabilizes cyclin D1-CDK4/6 complex and not necessarily inhibits their progesterone induced kinase activity. This confirms the studies done by Sherr & Roberts (1999). [20] Transcriptional activity of oncogenic E2F1 is regulated positively via TFs such as AP-1 or NF-κB and negatively by p53-p21 mediated pathway. In the current study, increased levels of mRNAs of NF-κB TFs by steroid hormones concurrently increased E2F1 mRNA level and decreased the levels of mRNAs of p53 and Rb in MCF-7 cells suggest that increased levels of E2F1 mRNA is via ERα tethering to NF-κB TFs caused the cell proliferation. Further, steroid hormone antagonists block the levels of mRNAs of NF-κB TFs as well as E2F1 by increasing p53 and Rb mRNA levels. These findings are similar to the results obtained by Dahlman-Wright et al., (2012)[21] and our previous study[13] where cell proliferation was caused by the induction E2F1 by estrogen via ERα-AP1 interaction. The ratio of pro-apoptotic and anti-apoptotic genes decides the fate of cell proliferation. In the present study, MCF-7 cells treated with steroid hormones showed increased levels of Bcl-2 mRNA and no change in the levels of Bax mRNA whereas, those treated with ICI 182,780 and RU486 showed increased levels of Bax mRNA and decreased levels of Bcl2 mRNA suggesting that steroid hormones increased anti-apoptotic mRNAs and lead to cell proliferation whereas their antagonists increased the levels of pro-apoptotic mRNAs which led to decrease in the cell proliferation confirm the hypothesis of Helmrich (2005). [22] However, MCF-7 cells treated with P4 alone showed increase in the level of Bcl-2 mRNA and did not show decreased level of p53 mRNA. This is in contrary to the results obtained by Formby and Wiley (1999) [23] suggested that P4 increases p53 mRNA by downregulating Bcl-2 and survivin mRNAs in breast cancer cells. The cross-talk of glucocorticoid with estrogen or progesterone plays a major role in the regulation of breast cancer. Dex antagonizes the effects of E2 or P4 by either occupying several estrogen binding regions/estrogen responsive element-like sites or overlapping with progesterone binding to progesterone responsive elements in the promoter of target genes. In the current study, MCF-7 cells when treated with E2+Dex and P4+Dex, Dex decreased the levels of E2-induced or P4-induced mRNA levels of cyclin D1, cyclin E1, E2F1 and Bcl-2 and increased levels of mRNAs of p53, Rb and Bax suggesting that Dex decreased the cell proliferation via antagonizing E2- or P4- mediated transcription of cyclins, oncogenic TFs and anti-apoptotic mRNAs. This is in agreement with our previous reports which showed Dex decreased cell proliferation. [13, 24] However, the role of cross-talk of Dex and E2 or P4 in the regulation of NF-κB TFs involved in cell cycle progression needs to be investigated. In conclusion, findings of the present study suggest that steroid hormones tether to NF-κB TFs similar to AP-1 tethering, via NRs cause cell proliferation through increasing the levels of mRNAs of cyclins and oncogenic E2F1. Also, Dex plays a dual role both as an agonist in the induction of NF-κB transcription factors, and as an antagonist in the expression pattern of cell cycle regulators, pro- and anti-apoptotic mRNAs. The antagonistic action of Dex is substrate and target specific and found to be more effective when used in combination with E2 than progesterone. Thus, glucocorticoids and their analogues could be used as potent antagonists of E2 in the therapeutic modality for treating triple positive breast cancer cells.

FUNDING/ACKNOWLEDGMENTS

The authors wish to express their gratitude to the Department of Science and Technology-Promotion of University Research and Scientific Excellence [SR/59/Z-23/2010/38(c)], and University Grant Commission Centre with Potential for Excellence in Particular Area (UGC-CPEPA) [8-2/2008(NS/PE)] New Delhi for providing financial support. The authors also express their gratitude to the Department of Microbiology and Biotechnology, Bangalore University for providing the DST-FIST, UGC-SAP and lab facility.

CONFLICT OF INTEREST

Conflict of interest declared none.

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We sincerely thank the above reviewers for peer reviewing the manuscript